ENRICHED CENTRAL NERVOUS SYSTEM STEM CELL AND PROGENITOR CELL POPULATIONS, AND METHODS FOR IDENTIFYING, ISOLATING AND ENRICHING FOR SUCH POPULATIONS

TECHNICAL FIELD

This invention relates generally to enriched neural stem cell and progenitor cell populations, and methods for identifying, isolating and enriching for neural stem and progenitor cells, particularly central nervous system neural stem cells and progenitor cells, and most particularly to enriched populations of neurosphere initiating cells (NS-IC).

BACKGROUND OF THE INVENTION

Stem cell populations constitute only a small percentage of the total number of cells in the body, but are of immense interest because of their ability to repopulate the body. The longevity of stem cells and the dissemination of stem cell progeny are desirable characteristics. There is significant commercial interest in these methods because stem cells have a number of clinical uses. There is also medical interest in the use of stem cells as a vehicle for gene therapy.

Proteins and other cell surface markers found on stem cell and progenitor cell populations are useful in preparing reagents for the separation and isolation of these populations. Cell surface markers are also useful in the further characterization of these important cells.

Neural stem cells have been isolated from the adult subventricular zone (SVZ) and hippocampus (Gage, (2000) Science *287*, 1433-38). These cells are an important source of new neurons, and offer the promise of novel central nervous system (CNS) repair therapies.

CNS stem cells are usually identified retrospectively by their ability to generate typical neurospheres or large adherent clones containing multiple neural cell types (Reynolds and Weiss, (1992) Science *255*, 1707-10; Davis and Temple, (1994) Nature *372*, 263-266; and Palmer *et al.*, (1997) Mol. Cell. Neurosci. *8*, 389-404), which precludes study of the initial stem cell population. Little is known about the unique biology of CNS stem cells, for example which

specific gene products they express. Identification of "unique" gene products expressed by CNS stem cells would expand the understanding of these important cells, aid in their identification *in vivo* and enable their positive enrichment *in vitro* for study and use.

Two different cell populations have recently been identified as including SVZ stem cells: GFAP-expressing astrocytes (Doetsch *et al.*, (1999) Cell *97* 703-16; Doetsch *et al.*, (1999) Proc. Natl. Acad. Sci. USA *96*, 11619-11624) and Notch1-expressing, ciliated ependymal cells lining the ventricles (Johansson *et al.*, (1999) Cell *96*, 25-34). These two distinct cell types are so intimately localized *in vivo* that it is difficult to separate them physically. Instead, defining specific features of stem cells will provide markers to help reveal their *in vivo* identity.

Genes expressed by adult CNS stem cells include Nestin, Musashi, Notch1 and GFAP (Sakakibara *et al.*, (1996) Devel Biol. *176*, 230-42; Johansson *et al.*, (1999) Cell *96*, 25-34; Doetsch *et al.*, (1999) Cell *97*, 703-16), but other CNS cell types also express these. Moreover, many of these markers are intracellular, limiting their usefulness for stem cell enrichment, although this problem can be overcome by creating transgenic mice with fluorescent reporter gene expression (Kawaguchi *et al.*, (2001) Mol. Cell. Neurosci. *17*, 259-273). A more generally useful marker would be a cell surface molecule allowing stem cell localization and purification from a wild-type mouse. Thus, there remains a need for tools, such as monoclonal antibodies that are useful in isolating and characterizing human non-hematopoietic progenitor and stem cells, and particularly central nervous system (CNS) neural stem cells and progenitor cells.

SUMMARY OF THE INVENTION

This invention provides methods for identifying, isolating, and enriching for human non-hematopoietic progenitor and stem cells, and particularly central nervous system (CNS) neural stem cells, progenitors, or combinations thereof which can initiate long-term neurospheres. The invention also provides for enriched populations containing CNS neural stem cells that can initiate neurospheres, and progenitor cells. As used herein, the term "neurosphere initiating cell (NS-IC)" refers to a cell that can initiate a long-term neurosphere culture. Those skilled in the art will recognize the NS-IC include stem cells or progenitors or a combination thereof, depending on the culture conditions used. A "neurosphere", in turn, is an aggregate or cluster of cells which includes neural stem cells and primitive progenitors. The identification, culture, growth, and use of neurospheres is disclosed in Weiss *et al.*, United States patent 5,750,376 and Weiss *et al.*, United States patent 5,851,832, both incorporated herein by reference. While the term "NS-IC"

is defined by the ability or capacity of that cell to form a neurosphere, these cells may also be appropriately grown in adherent culture (*see, for example,* Johe, United States patent 5,753,506, and Weiss, United States patent 5,750,376, which are both incorporated herein by reference). The methods and populations described herein are not to be limited to suspension cultures of NS-IC. A NS-IC is nestin$^+$ and has the capability to differentiate, under appropriate differentiating conditions, to neurons, astrocytes, and oligodendrocytes.

Enriched populations of non-hematopoietic stem cells and progenitor cells, preferably CNS neural stem cells and/or progenitors including NS-ICs, and methods of identifying, isolating, or enriching for such cells, are achieved by contacting a population of cells containing at least one stem cell or NS-IC or progenitor cell with a reagent that binds to a surface marker glycoprotein antigen ("CD49f antigen") recognized by an antibody that specifically binds to CD49f ("anti-CD49f antibody") or to a cell surface carbohydrate moiety ("CD15 antigen") recognized by an antibody that specifically binds to CD15 ("anti-CD15 antibody"). As used herein, the term "reagent" is meant to include any composition or compound that is capable of binding to, associating with, or recognizing an antigen. Examples of such reagents include, but are not limited to monoclonal antibodies, polyclonal antibodies, small molecules, receptors, ligands, proteins, protein fragments, polypeptides, polypeptide fragments, nucleic acids, nucleic acid fragments, antibody fragments, and any other "reagents" known to those skilled in the art.

While the methods described herein refer to the use of the CD49f antigen to enrich populations of neural cells for NS-IC, other cell surface markers found on CNS-SC may also be used. Examples of such cell surface markers include, but are not limited to, the CD133 antigen, which is recognized by anti-CD133 monoclonal antibodies such as monoclonal antibody AC133, and CD15, which is recognized by anti-CD15 monoclonal antibodies including, but not limited to MMA. Those skilled in the art will recognize that any of the methods described herein using the CD49f antigen and/or the anti-CD49f antibody may also be accomplished in conjunction with antibodies and/or antigens that recognize CD133 and/or CD15 antibodies and/or antigens. Those skilled in the art will also recognize that any other cell-surface marker present on neural stem cells, progenitors, or NS-IC can also be used in the methods of the instant invention. Moreover, the skilled artisan will recognize that any combination of CD49f, CD133 and/or CD15 antibodies and/or antigens can be used to produce populations enriched for NS-IC. Those skilled in the art will also recognize that any reference to anti-CD133, anti-CD15 and/or

anti-CD49f antibodies encompasses human, murine, rat, sheep, equine, goat, chicken, rabbit, guinea pig, and/or porcine antibodies.

The enriched populations of the invention may also be achieved by contacting a population of cells containing at least one stem cell or NS-IC or progenitor with a reagent that binds to the CD133 antigen including, but not limited to, the AC133 antibody. The contacting may be done before, during, and/or after the contacting with a reagent that binds to CD49f (*e.g.*, an anti-CD49f antibody) or to CD15 (*e.g.*, an anti-CD15 antibody). Antibodies to CD133 include, for example, monoclonal antibody AC133. As used herein, the terms "CD133 antibody" and "CD133 monoclonal antibody" encompass antibodies including, but not limited to AC133, that recognize the CD133 antigen. Moreover, $CD133^+$ cells are defined as cells containing the CD133 antigen.

In one preferred embodiment, the reagent is an anti-CD49f antibody (two such anti-CD49f antibodies are referred to herein as "GoH3" or "4F10"). In another preferred embodiment, the reagent is an anti-CD15 antibody (a preferred embodiment of anti-CD15 antibodies is referred to herein as MMA). Use of traditional techniques for cell sorting, such as by immunoselection (*e.g.*, FACS), permits identification, isolation, and/or enrichment for cells in which contact between the reagent and the CD49f antigen or the CD15 antigen has been detected.

This invention also provides methods of using an antibody to provide enriched populations of non-hematopoietic stem cells and progenitor cells, preferably CNS neural stem cells that can initiate neurospheres and progenitor cells, and that may be used in methods of identifying, isolating, or enriching for such cells, by contacting a population of cells containing at least one stem cell, NS-IC or progenitor cell with an anti-CD49f antibody or with an anti-CD15 antibody.

The cells of this invention, preferably the CNS neural stem cells, are additionally characterized as lacking cell surface markers for CD45 and CD34 (*e.g.*, $CD45^-$ and $CD34^-$).

This invention provides an antibody, herein called SC20, formerly known as 8G1 (Uchida, et al., PNAS 2000), which appears to recognize CD24 and permits subselection between populations of CNS neural stem cells (characterized as $SC20^{-/lo}$ or $CD24^{-/lo}$) and populations of CNS progenitor cells (characterized as $SC20^+$ or $CD24^{-/lo}$). CNS-SC isolated from fetal brains are $CD133^+SC20^{-/lo}$, which is also referred to as $CD133^+CD24^{-/lo}$. When CNS-SC are expanded *in vitro* as neurosphere cells, they may express CD24. Thus, the CD24 antigen appears to be upregulated as these cells proliferate. Therefore, neurosphere cells derived from

CNS-SC are heterogeneous for CD24 expression (low to high levels). Other antibodies that recognize CD24 include 32D12 [Diatec,Oslo, NORWAY (catalog number CD24 3061 - ab531)]; ALB9 [Accurate Chemical and Scientific Co., Westbury, NY; BEK, Miami, Florida; Biomeda Corporation, Foster City, CA; Biosource International, Camarillo, CA (catalog number AHS2402); Leinco Technologies, St. Louis, MO (catalog numbers C483; C484); Research Diagnostics, Inc., Flanders, NJ]; CLB134 [Accurate Chemical and Scientific Co., Westbury, NY; Cell Science, Norwood, MA (catalog number MON 1119)]; CLBGRANBLy1 [Accurate Chemical and Scientific Co., Westbury, NY; Research Diagnostics, Inc., Flanders, NJ]; SN3 [Caltag Laboratories, Inc., Burlingame, CA (catalog numbers MHCD2400; MHCD2401; MHCD2404)]; ML5 [BD Pharmingen, San Diego, CA (catalog numbers 555427; 555428; 555426)]; and 24C02 [Lab Vision Corporation, Freemont, CA (catalog number MS-1279); United States Biological, Swampscott, MA].

The invention involves methods for producing a population enriched for human CNS-SC and/or progenitors which can initiate neurospheres (NS-IC) by contacting neural or neural derived cells with a monoclonal antibody that binds to CD49f or with a monoclonal antibody that binds to CD15; selecting the cells that bind to this monoclonal antibody (*e.g.* $CD49f^{+}$, $CD15^{+}$, and $CD15^{-/lo}$ cells); and optionally removing the bound cells, wherein the selected cells are enriched for human CNS-SC and/or progenitors and wherein the $CD15^{-/lo}$ cells are a subset of the $CD133^{+}CD24^{-/lo}$ cells. The population containing neural or neural-dervided cells may be obtained from a neurosphere culture or an adherent culture or from primary neural tissue. In the various embodiments of this invention, the monoclonal antibody may be fluorochrome conjugated or may be conjugated to magnetic particles. Additionally, the selecting may be by fluorescence activated cell sorting, high gradient magnetic selection, or by attachment to and disattachment from the solid phase.

The methods may also involve the step of further enriching the population obtained from primary neural tissue for CNS-SC and/or progenitors by contacting the removed cells with a second monoclonal antibody SC20 and eliminating those cells that are $SC20^{+}$ ($CD24^{+}$) or $SC20^{hi}$ ($CD24^{hi}$) to produce a population enriched for CNS-SC and/or progenitors, wherein the selected cells in the population are $SC20^{-/lo}$ ($CD24^{-/lo}$). Alternatively, the selected cells can be further selected for those cells athat are $SC20^{-/lo}$ ($CD24^{-/lo}$).

The methods may also involve the step of further enriching the population for CNS-SC and/or progenitors by contacting the remaining cells with an anti-CD133 monoclonal antibody

and selecting those cells that bind to the anti-CD133 monoclonal antibody to obtain a population enriched for CNS-SC and/or progenitors. Alternatively, the neural or neural-derived cells may be contacted with an anti-CD133 monoclonal antibody (*e.g.*, AC133) prior to, during, or after contacting the cells with a monoclonal antibody that binds to CD49f or with a monoclonal antibody that binds to CD15. Throughout this specification, the term AntibodyX^+ is used interchangeably herein with the term AntibodyX^{hi}.

The invention involves methods for producing a population enriched for CNS-SC and/or progenitors, which can initiate neurospheres (NS-IC) or an adherent culture by selecting from a population of neural or neural-derived cells for cells that are $CD49f^+$. This may be accomplished by contacting the population with an anti-CD49f antibody, preferably, monoclonal antibody GoH3 or monoclonal antibody 4F10, and removing those cells that do not bind to monoclonal antibody GoH3 or monoclonal antibody 4F10. In one embodiment, the invention also provides a step for further enriching the population from primary neural tissues by removing the cells that are $CD24^+$ from the remaining population or by selecting for the cells that are $CD2^{-/lo}$. This may be done, for example, by selecting for cells that bind to monoclonal antibody SC20, which recognizes cells expressing high levels of CD24 (e.g. by removing the cells that bind to monoclonal antibody SC20, which recognizes CD24 ($CD24^+$ cells) or by selecting for cells that are $CD24^{-/lo}$). The remaining cells may be $CD24^{-/lo}$. Such methods can also involve the step of further enriching the population by selecting those cells that are $CD133^+$. Alternatively, the population of neural or neural-derived cells may be selected for $CD133^+$ cells prior to or concurrently with selecting for $CD49f^+$ cells. Cells that are $CD15^{hi}$ may be selected using monoclonal antibody MMA.

The invention involves methods for enriching from a population of neural cells for the populations of neurosphere initiating stem cells and/or progenitors (NS-IC) fraction by selecting from the neural cells for cells that bind to an anti-CD49f antibody, such as monoclonal antibody GoH3 or monoclonal antibody 4F10, (or to an anti-CD15 antibody such as monoclonal antibody MMA, *e.g.* cells that are $CD15^{-/lo}$ or $CD15^{hi}$, wherein the $CD15^{-/lo}$ cells are a subset of the $CD133^+CD24^{-/lo}$ population), wherein the selected cells are enriched in the fraction of NS-IC as compared with the population of neural cells. The fractions obtained from primary neural tissues can be further enriched by removing those cells that bind to an anti-CD24 antibody, such as monoclonal antibody SC20 (*e.g.*, by removing those cells that are $CD24^+$ such that the remaining cells may be $CD24^{-/lo}$ or by selecting for cells that are $CD24^{-/lo}$). Additionally, the fraction can be

further enriched by selecting for those cells that bind to an anti-CD133 antibody, such as monoclonal antibody AC133, those cells that bind to the anti-CD133 antibody, such as monoclonal antibody AC133 are selected prior to, during, or after selecting for those cells that bind to the anti-CD49f antibody, such as monoclonal antibody GoH3 or monoclonal antibody 4F10 (or to the anti-CD15 antibody, such as monoclonal antibody MMA).

The invention also provides methods for isolating a neurosphere initiating stem cell and/or progenitor cell (NS-IC) obtained from primary neural tissues, by selecting from a population of neural or neural-derived cells for cells that are $CD49f^{+}$ or that are $CD15^{hi}$ or $CD15^{-/lo}$, wherein the $CD15^{-/lo}$ cells are a subset of the $CD133^{+}CD24^{-/lo}$ cells; removing those cells that bind strongly to monoclonal antibody SC20 (*i.e.*, $SC20^{+}$ cells, wherein the remaining cells are $CD24^{-/lo}$ cells); introducing the remaining cells to a serum-free culture medium containing one or more growth factors selected from the group consisting of LIF, EGF, bFGF, and combinations thereof; and proliferating the remaining cells in the culture medium. The selected cells may be further enriched by selecting for cells that are $CD133^{+}$. This further enrichment step may be accomplished either before, during, or after selecting for cells that are $CD49f^{+}$, $CD15^{+}$, or $CD15^{-/lo}$.

Antibodies that specifically bind to the CD49f antigen are also provided, wherein the CD49f antigen specifically binds to the GoH3 antibody or the 4F10 antibody. This antibody may be produced by a hybridoma cell line. In some embodiments, this antibody may block simultaneous binding to the CD49f antigen by the antibody GoH3 and/or the 4F10 antibody.

Also provided are antibodies that specifically bind to the CD15 antigen, wherein the CD15 antigen specifically binds to the MMA antibody. This antibody may be produced by a hybridoma cell line. In some embodiments, this antibody may block simultaneous binding to the CD15 antigen by the antibody MMA.

Also provided is a method for the enrichment of human CNS-SC and/or progenitors which can initiate neurospheres (NS-IC) by combining a population of neural or neural-derived cells with a reagent that specifically binds to the CD49f antigen and/or to the CD15 antigen and selecting for those cells that bind to the CD49f reagent or the CD15 reagent, wherein the selected cells ($CD49f^{+}$, $CD15^{hi}$, or $CD15^{-/lo}$, wherein the $CD15^{-/lo}$ cells are a subset of the $CD133^{+}CD24^{-/lo}$ cells) are enriched for NS-IC. The reagent may include at least one antibody, and the at least one antibody may be fluorochrome conjugated, wherein the selecting is accomplished by flow cytometry. Alternatively, the at least one antibody may be conjugated to magnetic particles,

wherein the selecting is by high gradient magnetic selection. Such methods further involve the step of further enriching the population by combining the selected cells with a second reagent that specifically binds to the CD133 antigen and selecting for those cells that bind to the second reagent. The population of neural or neural-derived cells may be selected for cells that bind to a reagent that specifically binds to the CD133 antigen prior to, during, or following selecting for those cells that bind to a reagent that specifically binds to the CD49f antigen or to the CD15 antigen.

In any of the methods described herein, the population of cells can be further enriched for CNS-SC by either selecting for cells that are $CD24^{-/lo}$ or by removing cells that are $CD24^{+}$ from the population, such that the remaining cells are $CD24^{-/lo}$.

Methods for producing a population enriched for human CNS-SC and/or progenitors, which can initiate neurospheres (NS-IC) by selecting from a population of neural or neural derived cells for cells that are $CD49f^{+}$ or for cells that are $CD15^{+}$ or $CD15^{-/lo}$, wherein the $CD15^{-/lo}$ cells are a subset of the $CD133^{+}CD24^{-/lo}$ cells, are also provided.

Moreover, methods for producing a population enriched for human CNS-SC and/or progenitors which can initiate neurospheres (NS-IC) by selecting from neural or neural-derived cells for cells that bind to an anti-CD49f antibody, such as monoclonal antibody GoH3 or to monoclonal antibody 4F10, to produce a population enriched for CNS-SC, wherein the selecting is achieved by attachment to and disattachment from a solid phase. The population may be further enriched by selecting cells that bind to an anti-CD133 antibody, such as monoclonal antibody AC133. Additionally, the population may be further enriched by selecting for cells that bind to the anti-CD133 antibody (*e.g.* AC133) prior to, during, or after selecting for cells that bind to an anti-CD49f antibody, such as monoclonal antibodies GoH3 or 4F10.

The invention involves methods for producing a population enriched for human CNS-SC and/or progenitors which can initiate neurospheres (NS-IC) by selecting from neural or neural-derived cells for cells that bind to monoclonal antibody MMA, which recognizes the CD15 antigen (*e.g.* $CD15^{hi}$ or $CD15^{-/lo}$ cells, wherein the $CD15^{-/lo}$ cells are a subset of the $CD133^{+}CD24^{-/lo}$ cells), to produce a population enriched for CNS-SC, wherein the selecting is achieved by attachment to and disattachment from a solid phase. The population may be further enriched by selecting cells that bind to an anti-CD133 antibody, such as monoclonal antibody AC133. Additionally, the population may be further enriched by selecting for cells that bind to

an anti-CD133 antibody, such as monoclonal antibody AC133 prior to, during, or following selecting for cells that bind to a CD15 antibody, such as monoclonal antibody MMA.

Moreover, the invention provides methods for isolating a subset of human central nervous system progenitor cells by contacting neural or neural derived cells with a monoclonal antibody that binds to CD15 and selecting the neural or neural derived cells that bind to the monoclonal antibody (*e.g.* selecting for $CD15^{hi}$ or $CD15^{-/lo}$ cells, wherein the $CD15^{-/lo}$ cells are a subset of the $CD133^{+}CD24^{-/lo}$ cells) and optionally removing the bound cells (or the unbound cells), wherein the selected cells are a subset of human central nervous system progenitor cells that are selected from the group consisting of neuronal progenitors and glial progenitors. For example, the antibody may be monoclonal antibody MMA.

Finally, the invention involves methods for isolating a subset of human central nervous system progenitor cells by selecting from a population of neural or neural derived cells for those cells that are $CD15^{+}$ or $CD15^{-/lo}$, wherein the $CD15^{-/lo}$ cells are a subset of the $CD133^{+}CD24^{-/lo}$ cells and wherein the selected cells are a subset of human central nervous system progenitor cells that are selected from the group consisting of neuronal progenitors and glial progenitors.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only not intended to be limiting. Other features and advantages of the invention will be apparent from the following detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagram illustrating the proliferation and differentiation of a NS-IC.

FIG. 2 is a series of photographs showing that neurosphere cultures can be initiated from single-cell sorted $CD133^{+}$ cells.

FIG. 3 is a dot plot of FACS sorting data showing the isolation of human neural stem cells by cell surface markers. The figure shows that NS-IC typically express negative to low

levels of CD24 antigen for the SC20 (8G1) antibody. Since NS-IC expressed low levels of CD133 antigens, signals for CD133 detection was amplified in multi-step staining methods.

FIG. 4 is a dot plot of fluorescence activated cell sorting (FACS) data showing the isolation of human CNS neural stem cells using cell surface markers using monoclonal antibodies to CD49f alone or in conjunction with antibodies that recognize CD24. In Panel A, the x axis represents cells staining for antibodies to CD24, and the y axis represents cells staining for antibodies to CD133. In Panel B, the x axis represents cells staining for antibodies to CD49f, and the y axis represents cells staining for antibodies to CD133. In Panel C, the x axis represents cells staining for antibodies to CD49f, and the y axis represents cells staining for antibodies to CD24. In Panels D and E, the x axis represents cells staining for antibodies to CD24 and the y axis represents cells staining for antibodies to CD133.

FIG. 5 is a series of dot plots of fluorescence activated cell sorting (FACS) data showing the isolation of human CNS stem cells using cell surface markers using monoclonal antibodies to CD133 alone or in conjunction with antibodies that recognize CD49f, which shows that the majority of long-term neurosphere cells are $CD133^{+}CD49f^{+}$, the x axis represents cells staining for antibodies to CD49f and the y axis represents cells staining for antibodies to CD133.

FIG. 6 is a series of dot plots of fluorescence activated cell sorting (FACS) data showing the phenotypic analysis of human fetal brain cells. In Panel A, the x axis represents cells staining for antibodies to CD133 and the y axis represents cells staining for antibodies to CD15. In Panel B, the x axis represents cells staining for antibodies to CD24 and the y axis represents cells staining for antibodies to CD15. The area of detail represents a limiting dilution of $CD15^{+}CD24^{-/lo}$ cells of 1 in 4.5 (22.5%). In Panel C, the x axis represents cells staining for antibodies to CD24 and the y axis represents cells staining for antibodies to CD133. As shown in Panels B and C, $CD133^{+}CD24^{-/lo}$ cells consist of $CD15^{hi}CD24^{-/lo}$ and $CD133^{+}CD15^{-/lo}CD24^{-/lo}$ cells.

FIG. 7 is a diagram showing that CD15 expression defines different subsets of expandable CNS stem cells and progenitors. Panel A shows a schematic diagram of experimental design. Panel B shows the proportion of NS-IC activity in different subsets. 80.56%, 11.81%, and 7.35% of neurospheres were derived from the $CD133^{+}CD15^{+}CD24^{-/lo}$, $CD133^{+}CD15^{-/lo}CD24^{-/lo}$, and $CD133^{+}CD15^{+}CD24^{+}$ subsets, respectively.

FIG. 8 shows the immunohistochemistry of NOD-Scid brain engrafted with $CD133^{+}CD15^{-/lo}CD24^{-/lo}$ sorted/expanded neurosphere cells in the olfactory bulb (A) and $CD49f^{+}CD24^{-/lo}$ sorted/expanded neurosphere cells in the hippocampus (B). Neurosphere cells

were transplanted into the lateral ventricles of neonatal NOD-Scid and grafts were harvested 6 months after transplantation.

DETAILED DESCRIPTION OF THE INVENTION

A population of cells exists within the adult central nervous system (CNS), which exhibit stem cell properties. They have the ability to self-renew and to produce the differentiated mature cell phenotypes of the adult CNS. These stem cells are found throughout the CNS, and particularly in the subventricular regions, and dentate gyrus of the hippocampus.

Growth factor-responsive stem cells can be isolated from many regions of the neuraxis and at different stages of development, of murine, rodent and human CNS tissue. These cells vary in their response to growth factors such as EGF, basic FGF (bFGF, FGF-2) and transforming growth factor alpha (TGFα), and can be maintained and expanded in culture in an undifferentiated state for long periods of time. Both adult and embryonic murine progenitor cells respond to EGF and grow as spheres of undifferentiated cells. These cells show the characteristics of stem cells in that they are multipotent, and under serum containing conditions can differentiate into neurons, astrocytes and oligodendrocytes, as well as maintaining a subpopulation, which remains undifferentiated and continues to proliferate under EGF administration. Murine EGF-responsive progenitor cells express mRNA for the EGF receptor *in vitro*. Human CNS neural stem cell cultures have also been identified. The identification, culture, growth, and use of mammalian, including human, neural stem cell cultures, either as suspension cultures or as adherent cultures, is disclosed in Weiss *et al.*, United States patent 5,750,376 and Weiss *et al.*, United States patent 5,851,832, both incorporated herein by reference. Similarly, Johe, United States patent 5,753,506, also incorporated herein by reference, refers to adherent CNS neural stem cell cultures. When cultured in suspension, CNS neural stem cell cultures typically form neurospheres.

FIG. 1 shows the proliferation of a NS-IC as it develops into a neurosphere, and the subsequent differentiation into neuronal and glial phenotypes, as well as the generation of a progeny NS-IC. In the presence of one or more proliferation-inducing growth factors, the NS-IC divides and gives rise to a sphere of undifferentiated cells composed of more stem cells and progenitor cells (a "neurosphere"). When the clonally derived neurosphere is dissociated and plated as single cells in the presence of one or more proliferation-inducing growth factors, each

NS-IC can generate a new neurosphere. The cells of a single neurosphere are clonal in nature because they are the progeny of a single neural stem cell. In the continued presence of a proliferation-inducing growth factor such as EGF or the like, precursor cells within the neurosphere continue to divide resulting in an increase in the size of the neurosphere and the number of undifferentiated neural cells. The neurosphere is not immunoreactive for neurofilament (NF; a marker for neurons), neuron-specific enolase (NSE; a marker for neurons), glial fibrillary acidic protein (GFAP; a marker for astrocytes), or myelin basic protein (MBP; a marker for oligodendrocytes). However, cells within the neurosphere are immunoreactive for nestin, an intermediate filament protein found in many types of undifferentiated CNS cells (Lehndahl *et al.*, 60 CELL 585-595 (1990), incorporated herein by reference). Antibodies are available to identify nestin, including the rat antibody referred to as Rat401. If the neurospheres are cultured in conditions that allow differentiation, the progenitor cells differentiate to neurons, astrocytes and oligodendrocytes. The mature phenotypes associated with the differentiated cell types that may be derived from the neural stem cell progeny are predominantly negative for the nestin phenotype.

The terminology used for undifferentiated, multipotent, self-renewing, neural cells has evolved such that these cells are now termed "neural stem cells." A neural stem cell is a clonogenic multipotent stem cell, which is able to divide and, under appropriate conditions, has self-renewal capability for NS-IC and can include in its progeny daughter cells, which can terminally differentiate into neurons, astrocytes, and oligodendrocytes. Hence, the neural stem cell is "multipotent" because stem cell progeny have multiple differentiation pathways. A neural stem cell is capable of self-maintenance, meaning that with each cell division, one daughter cell will also be on average a stem cell.

The non-stem cell progeny of a neural stem cell are typically referred to as "progenitor" cells, which are capable of giving rise to various cell types within one or more lineages. The term "neural progenitor cell" refers to an undifferentiated cell derived from a neural stem cell, and is not itself a stem cell. Some progenitor cells can produce progeny that are capable of differentiating into more than one cell type. For example, an O-2A cell is a glial progenitor cell that gives rise to oligodendrocytes and type II astrocytes, and thus could be termed a "bipotential" progenitor cell. A distinguishing feature of a progenitor cell is that, unlike a stem cell, it does not exhibit self maintenance, and, typically, is thought to be committed to a

particular path of differentiation and will, under appropriate conditions, eventually differentiate into glia or neurons.

As used herein, the term "precursor cells" refers to the progeny of neural stem cells, and thus includes both progenitor cells and daughter neural stem cells.

Cell markers. This invention provides for the identification, isolation, enrichment, and culture of neural stem cells and/or progenitors that are capable of forming neurospheres (NS-IC). NS-ICs are identified or selected through the binding of antigens, found on the surfaces of NS-ICs, to reagents that specifically bind the cell surface antigen.

In order to normalize the distribution to a control, each cell is recorded as a data point having a particular intensity of staining. These data points may be displayed according to a log scale, where the unit of measure is arbitrary staining intensity. In one example, the brightest cells in a population are designated as 3 logs more intense than the cells having the lowest level of staining. When displayed in this manner, it is clear that the cells falling in the highest log of staining intensity are bright, while those in the lowest intensity are negative. The "low" staining cells, which fall in the 2-3 log of staining intensity, may have properties that are unique from the negative and positive cells. An alternative control may utilize a substrate having a defined density of marker on its surface, for example a fabricated bead or cell line, which provides the positive control for intensity. The "low" designation indicates that the level of staining is above the brightness of an isotype-matched control, but is not as intense as the most brightly staining cells normally found in the population.

As used herein, the terms $CD15^{lo}$ and/or $CD15^{low}$ and/or $CD15^{-/lo}$ refer to "low" staining cells, which fall into the 1^{st}-2^{nd} log of staining intensity. When the few molecules (<100-500) in a given antigen were expressed on the cell surface, the signal to noise ratio may be poor to determine whether a given antigen is expressed on the cell surface. Those skilled in the relevant arts will recognize that any of the antibodies described herein can also be described using the "lo" or "low" designation (*i.e.* antibodyX^{lo} or antibodyX^{low}), without altering the intended meaning. Likewise, as used herein, the terms $CD15^{hi}$, $CD15^{high}$, and/or $CD15^{bright}$ refer to those cells in the population designated as 3 logs more intense than the cells having the lowest level of staining. Again, those skilled in the art will recognize that any antibody can be described using these designations, without altering the intended meaning (*i.e.*, antibodyX^{hi}, antibodyX^{high}, or antibodyX^{bright}). The designation antibodyX^{med} is intended to refer to an antibody having a

staining intensity falling between "low" and "bright". Moreover, as used herein, the designations antibodyX^+ and antibodyX^{hi} are used interchangeably.

One of the antigens found on the surface of NS-IC is an antigen that binds to the AC133 monoclonal antibody (*i.e.*, the CD133 antigen). Yin *et al.*, United States patent 5,843,633, incorporated herein by reference, describes a monoclonal antibody called AC133, which binds to a surface marker glycoprotein on hematopoietic stem and progenitor cells. The AC133 antigen (also referred to herein as the "CD133 antigen" or "CD133") is a 5-transmembrane cell surface antigen with a molecular weight of 117 kDa. Expression of this antigen is highly tissue specific, and has been detected on a subset of hematopoietic progenitor cells derived from human bone marrow, fetal bone marrow and liver, cord blood, and adult peripheral blood. The subset of cells recognized by the AC133 antibody is $CD34^{bright}$, and contains substantially all of the CFU-GM activity present in the $CD34^+$ population, making AC133 useful as a reagent for isolating and characterizing human hematopoietic progenitor and stem cells.

The AC133 antibody (also referred to herein as the 5F3 antibody) is exemplary of antibody embodiments of reagents that recognize a human cell marker termed prominin. Prominin is a polytopic membrane protein expressed in various epithelial cells (Weigmann *et al.*, 94(23) Proc Natl Acad Sci U S A. 12425-30 (1997); Corbeil *et al.*, 112 (Pt 7) J Cell Sci. 1023-33 (1999); Corbeil *et al.*, 91(7) Blood 2625-6 (1998); Miriglia *et al.*, 91(11) Blood 4390-1 (1998)). Various AC133 antibodies are described in United States patent 5,843,633, which is incorporated herein by reference. A deposit of the murine hybridoma cell line AC133 was made at the American Type Tissue Collection, 12301 Parklawn Drive, Rockville MD 20852, on Apr. 24, 1997, and given the ATCC designation HB12346. These AC133 antibodies are capable of immunoselection for a subset of human cells of interest in this invention. Preferred AC133 monoclonal antibodies can be obtained commercially from Miltenyi Biotec Inc. (Auburn CA), including, but not limited to, AC133/1-PE antibody (Cat #808-01) and AC133/2-PE antibody (Cat #809-01). For MACS separation, a 50:50 mixture of the monoclonal antibodies is preferred. The high tissue specificity of AC133 expression is particularly advantageous during enrichment for highly purified NS-IC populations. A discussion of the use of the AC133 antigen to select NS-IC is found in United States Patent No. 6,468,794, which is incorporated herein by reference.

"Anti-CD133 antibodies" are characterized by binding to the CD133 protein in native, in FACS and immunoprecipitation experiments, as well as denatured, in western blot experiments, conformation. The CD133 antigen has been reported to have several reduced molecular weights

in the range of 125 kDa to 127 kDa according to United States Patent No. 5,843,633 and 115 kDa to 127 kDa according to United States Published Patent Application No. 20010051372. Examples of anti-CD133 antibodies include, but are not limited to, AC133 and SC111 (StemCells, Inc., Palo Alto, CA).

CD45 is the T200/leucocyte common antigen. Antibodies to CD45 are commercially available from, *e.g.* Miltenyi Biotec (Auburn, CA) (catalog numbers 130-080-201; 130-080-202); and Research Diagnostics (Flanders, NJ) (catalog numbers RDI-M1343clb; RDI-CBL124; RDI-CBL148; RDI-CBL464, *etc.*). In a preferred embodiment, the cells of this invention and cultures containing them, are additionally characterized (in addition to being prominin positive) as lacking cell surface markers such as CD45.

CD34 is also known as gp105-120. Monoclonal antibodies to CD34 are commercially available from, *e.g.*, Miltenyi Biotec (Auburn, CA) (catalog numbers 130-090-954); Research Diagnostics (Flanders, NJ) (catalog numbers RDI-M1636clb; RDI-CBL128; RDI-CBL496FT; RDI-M2281clb; RDI-CD34-581, etc.); BD Biosciences, Pharmingen (San Diego, CA) (catalog number 550760)). Anti-CD34 monoclonal antibodies have been used to quantify and purify lymphohematopoietic stem/progenitor cells for research and for clinical bone marrow transplantation. CD34 is a monomeric cell surface antigen with a molecular mass of approximately 110 kDa that is selectively expressed on human progenitor cells. The gene is expressed by small vessel endothelial cells in addition to hematopoietic progenitor cells and is a single-chain 105-120 kDa heavily O-gylcosylated transmembrane glycoprotein. The sequence is disclosed by Simons et al. (1992) J. Immun. 148:267-271.

The monoclonal antibody SC20, formerly known as 8G1 (Uchida et al., PNAS 2000) is believed to recognize CD24. It specifically reacts with the 515 kilodalton α-chain of human LRP/A2MR which is expressed in a restricted spectrum of cell types. A strong immunohistochemical reaction is seen in hepatocytes, tissue macrophages, subsets of neurons and astrocytes in the central nervous system, fibroblasts, smooth muscle cells, and monocyte-derived foam cells in atherosclerotic lesions in the arterial wall. This antibody can also be used for the characterization of a subset of myelomonocytic subtypes of chronic and acute leukemia (CD91). Antibodies to CD91 are commercially available from, *e.g.*, Research Diagnostics (Flanders, NJ) (catalog numbers RDI-PRO651102; RDI-PRO610102; RDI-PRO61065, *etc.*).

Other examples of antibodies that recognize CD24 include 32D12 [Diatec, Oslo, NORWAY (catalog number CD24 3061 - ab531)]; ALB9 [Accurate Chemical and Scientific

Co., Westbury, NY; BEK, Miami, Florida; Biomeda Corporation, Foster City, CA; Biosource International, Camarillo, CA (catalog number AHS2402); Leinco Technologies, St. Louis, MO (catalog numbers C483; C484); Research Diagnostics, Inc., Flanders, NJ]; CLB134 [Accurate Chemical and Scientific Co, Westbury, NY; Cell Sciences, Norwood, MA (catalog number MON 1119)]; CLBGRANBLyl [Accurate Chemical and Scientific Co., Westbury, NY; Research Diagnostics, Inc., Flanders, NJ]; SN3 [Caltag Laboratories, Inc., Burlingame, CA (catalog numbers MHCD2400; MHCD2401; MHCD2404)]; ML5 [BD Pharmingen, San Diego, CA (catalog numbers 555427; 555428; 555426)]; and 24C02 [Lab Vision Corporation, Freemont, CA (catalog number MS-1279); United States Biological, Swampscott, MA].

Those skilled in the art will recognize that the designations $SC20^{+}$ and $CD24^{+}$ as well as $SC20^{-/lo}$ and $CD24^{-/lo}$ are synonymous and are used interchangeably throughout this application. CNS-SC isolated from fetal brains are $CD133^{+}SC20^{-/lo}$ cells (*e.g.* the cells express low levels of CD24). When CNS-SC are expanded *in vitro* as neurosphere cells, they may express CD24. The CD24 antigen appears to be upregulated as cells proliferate. Therefore, neurosphere cells derived from CNS-SC are heterogeneous for CD24 express (low levels to high). Such cells are also $CD133^{+}$.

CD49f (also known as integrin alpha-6) (GenBank Accession No. X53586; SWISSPROT Accession No. P23229) is a 150 kDa transmembrane protein that is part of an integrin heterodimer expressed predominantly by epithelial cells. Integrin alpha-6 associates with the integrin β-1 (CD29) chain to form VLAA-6 and with the integrin β-4 chain to form the laminin and kalinin receptors. CD49f is expressed mainly on T cells, monocytes, platelets, epithelial and endothelial cells, perineural cells, and trophoblasts of placenta. The sequence of CD49f may be found in, *e.g.*, Tamura et al., J. Cell Biol. 111:1593-604 (1990), which is incorporated herein by reference. There are two alternatively spliced forms of CD49f cDNA, which have been described as having different cytoplasmic domains. The A form alone is expressed in the lung, liver, spleen, and cervix. Only the B form is observed in the brain, ovary, and kidney, and both forms have been detected in other tissues. CD49f/CD29 α6β1 is the laminin receptor on platelets, monocytes, and T lymphocytes, and CD49f/CD29-mediated T cell binding to laminin provides a co-stimulatory signal to T cells for activation and proliferation.

Antibodies to CD49f have not been used in methods for identifying, isolating, or enriching for non-hematopoietic stem cells or progenitor cells, particularly central nervous system (CNS) neural stem cells and progenitor cells.

The sequence of CD49f is presented below in Table A. Alpha-6 associates with the integrin β-1 (CD29) chain to form VLAA-6 and with the integrin β-4 claim to form the laminin and kalinin receptors. Antibodies that recognize CD49f include GoH3 [Research Diagnostics, Inc., Flanders, NJ (catalog numbers RDI-M1566 and RDI-M1672clb); BD Biosciences (www.bdbiosciences.com) (catalog numbers 55710, 557511, 551140, 551129, 555734, 555735, 555736) ; and ICN Biomed (www.incbiomed.com)] and 4F10 [Research Diagnostics, Inc., Flanders, NJ (catalog number RDI-CBL458)].

TABLE A: SEQUENCE OF CD49f (SEQ ID NO:1)

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   1 maaagqlcll ylsagllsrl gaafnldtre dnvirkygdp gslfgfslam hwqlqpedkr
  61 lllvgaprge alplqranrt gglyscdita rgpctriefd ndadptsesk edqwmgvtvq
 121 sqgpggkvvt cahryekrqh vntkqesrdi fgrcyvlsqn lrieddmdgg dwsfcdgrlr
 181 ghekfgscqq gvaatftkdf hyivfgapgt ynwkgivrve qknntffdmn ifedgpyevg
 241 getehdeslv pvpansylgl lfltsvsytd pdqfvyktrp preqpdtfpd vmmnsylgfs
 301 ldsgkgivsk deitfvsgap ranhsgavvl lkrdmksahl lpehifdgeg lassfgydva
 361 vvdlnkdgwq divigapqyf drdgevggav yvymnqqgrw nnvkpirlng tkdsmfgiav
 421 knigdinqdg ypdiavgapy ddlgkvfiyh gsangintkp tqvlkgispy fgysiagnmd
 481 ldrnsypdva vgslsdsvti frsrpviniq ktitvtpnri dlrqktacga psgiclqvks
 541 cfeytanpag ynpsisivgt leaekerrks glssrvqfrn qgsepkytqe ltlkrqkqkv
 601 cmeetlwlqd nirdklrpip itasveiqep ssrrrvnslp evlpilnsde pktahidvhf
 661 lkegcgddnv cnsnlkleyk fctregnqdk fsylpiqkgv pelvlkdqkd ialeitvtns
 721 psnprnptkd gddaheakli atfpdtltys ayrelrafpe kqlscvanqn gsqadcelgn
 781 pfkrnsnvtf ylvlsttevt fdtpdldinl klettsnqdn lapitakakv vielllsvsg
 841 vakpsqvyfg gtvvgeqamk sedevgslie yefrvinlgk pltnlgtatl niqwpkeisn
 901 gkwllylvkv eskglekvtc epqkeinsln lteshnsrkk reitekqidd nrkfslfaer
 961 kyqtlncsvn vncvnircpl rgldskasli lrsrlwnstf leeysklnyl dilmrafidv
1021 taaaenirlp nagtqvrvtv fpsktvaqys gvpwwiilva ilagilmlal lvfilwkcgf
1081 fkrsryddsv pryhavrirk eereikdeky idnlekkqwi tkwnrnesys
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CD15 (also known as Lewis X, or LeX) (GenBank Accession No. NM 002033) is a 220 kDa branched pentasaccharide. The CD15 carbohydrate epitope is expressed in mature human neutrophils, monocytes, and eosinophils, as well as in adult mouse subventricular zone (SVZ) stem cells. It can also be found present on embryonic tissues and adenocarcinomas, myeloid leukemias and Reed-Sternberg cells. In such tissues, the Lewis X epitope is considered to be involved in cell-cell interactions. CD15 is carried by the CD11/CD18 and CD66 glycoproteins. CD15 antibodies recognize the terminal trisaccharide structure Galβ1→4[Fucα1→3]GlcNAc (LeX antigen). The majority of the CD15 antibodies are IgM, and they do not cross react with the sialylated form of CD15, CD15s.

CD15 is a fucose-containing trisaccharide widely distributed in many tissues and is developmentally expressed in some rodent and human tissues, *i.e.*, brain and lung, and mouse early embryo. Additionally, CD15 is present on the surface of pluripotent stem cells, such as

mouse embryonic stem cells and primordial germ cells. The sequence of CD15 is presented in Table B. CD15 is useful as a cell type marker since it allows for stem cell localization and purification. Antibodies that recognize human CD15 include MMA (BD Biosciences (www.bdbiosciences.com) (catalog numbers 340703, 340850, 347420, 347423, 559045)).

Cell surface carbohydrate moieties are useful cell type markers (Jessell et al., (1990) Ann. Rev. Neurosci *13*, 227-55). The LeX antigen, which is the trisaccharide 3-fucosyl-N-acetyllactosamine or FAL (Gooi et al., (1981) Nature *292*, 156-58), also known as SSEA-1 (stage specific embryonic antigen 1) or CD15 (leukocyte cluster of differentiation 15), is highly expressed on pluripotent stem cells: it is found on mouse and human embryonic carcinoma cells, mouse pre-implantation embryos, embryonic stem cells, teratocarcinoma cells and primordial germ cells (Solter and Knowles, (1978) Proc. Natl. Acad. Sci. USA *75*, 5565-69; Fox et al., (1981) Dev. Biol. *83*, 391-98; Bird and Kimber, (1984) Dev. Biol. *104*, 449-60; Muramatsu, (1994) Nagoya J. Med. Sci. *57*, 95-108; Marani et al., (1986) Acta. Morphol. Neerl. Scand. *24*, 103-110; Gomperts et al., (1994) Development *120*, 135-41). Intriguingly, CNS cell sub-populations in various species also express this marker during development and in adulthood. LeX is expressed in germinal zones in the murine embryonic telencephalon (Yamamoto et al., (1985) Proc. Natl. Acad. Sci. USA *82*, 3045-49; Allendoerfer et al., (1995) Mol. Cell. Neurosci. *6*, 381-95; Allendoerfer et al., (1999) Dev. Biol. *211*, 208-19; Tole et al., (1995) J. Neurosci *15*, 624-27; Ashwell and Mai, (1997) Cell Tissue Res. *289*, 17-23) and spinal cord (Dodd and Jessell, (1986) J. Exp. Biol. *129*, 225-38), and in the cerebellar external granular layer (Marani and Tetteroo, (1983) Histochemistry *78*, 157-61. In the adult mouse CNS, LeX is expressed by sub-populations of astrocytes, tanycytes, and a few neurons (Bartsch and Mai, (1991) Cell Tissue Res. *263*, 353-66; Gocht et al., (1996) Histol. Histopathol. *11*, 1007-28; Ashwell and Mai, (1997) Cell Tissue Res. *289*, 17-23).

TABLE B: SEQUENCE OF CD15 (SEQ ID NO:2)

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   1 ctgctcctgc gcggcagctg ctttagaagg tctcgagcct cctgtacctt cccagggatg
  61 aaccgggcct tccctctgga aggcgagggt tcgggccaca gtgagcgagg gccagggcgg
 121 tgggcgcgcg cagagggaaa ccggatcagt tgagagagaa tcaagagtag cggatgaggc
 181 gcttgtgggg cgcggcccgg aagccctcgg gcgcgggctg ggagaaggag tgggcggagg
 241 cgccgcagga ggctcccggg gcctggtcgg gccggctggg ccccgggcgc agtggaagaa
 301 agggacgggc ggtgcccggt tgggcgtcct ggccagctca ccttgccctg gcggctcgcc
 361 ccgcccggca cttgggagga gcagggcagg gcccgcggcc tttgcattct gggaccgccc
 421 ccttccattc ccgggccagc ggcgagcggc agcgacggct ggagccgcag ctacagcatg
 481 agagccggtg ccgctcctcc acgcctgcgg acgcgtggcg agcggaggca gcgctgcctg
 541 ttcgcgccat gggggcaccg tggggctcgc cgacggcggc ggcgggcggg cggcgcgggt
 601 ggcgccgagg ccgggggctg ccatggaccg tctgtgtgct ggcggccgcc ggcttgacgt
 661 gtacggcgct gatcacctac gcttgctggg ggcagctgcc gccgctgccc tgggcgtcgc
 721 caaccccgtc gcgaccggtg ggcgtgctgc tgtggtggga gcccttcggg gggcgcgata
 781 gcgccccgag gccgcccccc gactgccggc tgcgcttcaa catcagcggc tgccgcctgc
 841 tcaccgaccg cgcgtcctac ggagaggctc aggccgtgct tttccaccac cgcgacctcg
 901 tgaaggggcc ccccgactgg cccccgcccc ggggcatcca ggcgcacact gccgaggagg
 961 tggatctgcg cgtgttggac tacgaggagg cagcggcggc ggcagaagcc ctggcgacct
1021 ccagccccag gcccccgggc cagcgctggg tttggatgaa cttcgagtcg ccctcgcact
1081 ccccggggct gcgaagcctg gcaagtaacc tcttcaactg gacgctctcc taccgggcgg
1141 actcggacgt ctttgtgcct tatggctacc tctaccccag aagccacccc ggcgacccgc
1201 cctcaggcct ggccccgcca ctgtccagga aacaggggct ggtggcatgg gtggtgagcc
1261 actgggacga gcgccaggcc cgggtccgct actaccacca actgagccaa catgtgaccg
1321 tggacgtgtt cggccggggc gggccggggc agccggtgcc cgaaattggg ctcctgcaca
1381 cagtggcccg ctacaagttc tacctggctt tcgagaactc gcagcacctg gattatatca
1441 ccgagaagct ctggcgcaac gcgttgctcg ctggggcggt gccggtggtg ctgggcccag
1501 accgtgccaa ctacgagcgc tttgtgcccc gcggcgcctt catccacgtg gacgacttcc
1561 caagtgcctc ctccctggcc tcgtacctgc ttttcctcga ccgcaacccc gcggtctatc
1621 gccgctactt ccactggcgc cggagctacg ctgtccacat cacctccttc tgggacgagc
1681 cttggtgccg ggtgtgccag gctgtacaga gggctgggga ccggcccaag agcatacgga
1741 acttggccag ctggttcgag cggtgaagcc gcgctcccct ggaagcgacc caggggaggc
1801 caagttgtca gctttttgat cctctactgt gcatctcctt gactgccgca tcatgggagt
1861 aagttcttca aacacccatt tttgctctat gggaaaaaaa cgatttacca attaatatta
1921 ctcagcacag agatgggggc ccggtttcca tatttttgc acagctagca attgggctcc
1981 ctttgctgct gatgggcatc attgtttagg ggtgaaggag ggggttcttc ctcaccttgt
2041 aaccagtgca gaaatgaaat agcttagcgg caagaagccg ttgaggcggt ttcctgaatt
2101 tccccatctg ccacaggcca tatttgtggc ccgtgcagct tccaaatctc atacacaact
2161 gttcccgatt cacgtttttc tggaccaagg tgaagcaaat ttgtggttgt agaaggagcc
2221 ttgttggtgg agagtggaag gactgtggct gcaggtggga ctttgttgtt tggattcctc
2281 acagccttgg ctcctgagaa aggtgaggag ggcagtccaa gaggggccgc tgacttcttt
2341 cacaagtact atctgttccc ctgtcctgtg aatggaagca aagtgctgga ttgtccttgg
2401 aggaaactta agatgaatac atgcgtgtac ctcactttac ataagaaatg tattcctgaa
2461 aagctgcatt taaatcaagt cccaaattca ttgacttagg ggagttcagt atttaatgaa
2521 accctatgga gaatttatcc ctttacaatg tgaatagtca tctcctaatt tgtttcttct
2581 gtctttatgt ttttctataa cctggatttt ttaaatcata ttaaaattac agatgtgaaa
2641 ataaagcaga agcaaccttt ttccctcttc ccagaaaacc agtctgtgtt tacagacaga
2701 agagaaggaa gccatagtgt cacttccaca caattattta tttcatgtct ttactggacc
2761 tgaaatttaa actgcaatgc cagtcctgca ggagtgctgg cattaccctc tgcagaacag
2821 tgaaaggtat tgcactacat tatggaatca tgcaaaaaaa a
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At least two different subsets of $CD133^{+}SC20^{-/lo}$ cells exist: those that are $CD15^{-/lo}$ and those that are $CD15^{hi}$. Both subsets expanded to give rise to neurospheres. It is unclear whether $CD15^{-/lo}$ or $CD15^{hi}$ cells are more primitive CNS-SC. Neurosphere cells derived from the $CD15^{-/lo}$ subset of cells engrafted well in the NOD-SCID mouse.

Biological significance of CD15 (LeX)

LeX is expressed on embryonic pluripotent stem cells and on adult CNS stem cells. LeX influences blastocyst adhesion, (Bird, J.M. et al., (1984) Dev. Biol. *104*, 449-460; Hakomori, S.I. (1992) Histochem. J. *24*, 771-776), and it can influence CNS stem cell adhesion. Carbohydrate ectodomains on proteoglycans can be shed into the extracellular matrix where they interact with growth factors (Kato, M. et al., (1998) Nat. Med. *4,* 691-697). LeX is present in the extracellular matrix (Gocht, A. et al., (1996) Histol. Histopathol. *11*, 1007-1028) and shedding of LeX^{+} material by adult SVZ cells *in vitro* and diffuse LeX staining in neurogenic zones has been observed. Low concentrations of free LeX can promote FGF2 oligomerization and stimulate its mitogenicity for embryonic stem cells (Milev, P. et al., (1998) J. Biol. Chem. *273*, 21439-21442; Jirmanova, L. et al., (1999) Int. J. Dev. Biol. *43,* 555-562). However, excess LeX inhibits FGF2 mitogenicity (Dvorak, P. et al., (1998) J. Cell Science *111*, 2945-2952). Thus, different concentrations of LeX in the extracellular environment can regulate growth factor access to, and influence on, CNS stem cells. In the embryo, LeX-containing carbohydrates can bind Wnts, and they may continue to bind critical growth modulators in the adult. The large carrier molecule for LeX identified in the developing CNS and adult neurogenic regions may be important to its regulative role.

Isolation of subsets of stem and progenitor cells

Establishing a hierarchy of a particular cell fate map has now been accomplished for the mouse hematopoietic stem cells and its progeny. This fate mapping uses the techniques that have been applied in this invention and can be found more descriptively in Morrison SJ, Weissman IL. Immunity 1994 Nov;1(8):661-73; Kondo M, Weissman IL, Akashi K. Cell 1997 Nov 28;91(5):661-72; Akashi K, Traver D, Miyamoto T, Weissman IL. Nature 2000 Mar 9;404(6774):193-7. The further dissection of the initially described mouse hematopoietic stem cell population was accomplished by using surface phenotypes to subdivide the hematopoietic stem cell population into both a short and long term repopulating fraction. This technology was then applied to the progeny of the hematopoietic stem cells to identify a lymphomyeloid progenitor; a myeloid restricted progenitor, and a common lymphoid progenitor.

Isolation of subsets of NS-IC

NS-IC are obtained from a cell population isolated from neural tissues (typically fetal brain tissue), prior to expansion *in vitro*. As a result, the NS-IC population can be $CD133^{+}CD24^{-/lo}$ or $CD49f^{+}CD24^{-/lo}$. Following *in vitro* expansion, this population of cells may be $CD24^{hi}$. NS-IC obtained following culture can be classified as $CD133^{+}CD49f^{+}$.

The invention provides for selection methodologies using the cellular marker CD49f that can be used to isolate subsets of NS-IC (including stem cells and progenitors). Isolation of such subsets can be performed either before or after selection of $CD133^{+}$ cells and/or $CD49f^{+}$ cells. CD15 can be used to isolate $CD15^{hi}CD24^{-/lo}$ and $CD133^{+}CD15^{-/lo}CD24^{-/lo}$ cell populations, which are enriched for NS-IC. As shown in Fig. 7, CD15 expression defines different subsets of expandable CNS stem cells and progenitors. As shown in Example 6, $CD15^{-/lo}$ cells have the ability to generate neurospheres and engraft well following transplantation.

Cell Deposits. The 8G1.7 cultures (now known as SC20) have been deposited with ATCC, 10801 University Blvd., Manassas, VA 20110-2209, under ATCC accession numbers PTA-993 and PTA-994, respectively, in accordance with the provisions of the Budapest Treaty for the Deposit of Microorganisms. As noted in United States patent 5,843,633, the murine hybridoma cell line AC133 was deposited at the American Type Tissue Collection, 12301 Parklawn Drive, Rockville, MD 20852 (ATCC designation HB12346) in accordance with the provisions of the Budapest Treaty.

Anti-CD49f and anti-CD15 antibodies are commercially available.

Isolation, enrichment, and selection of cells. The population of cells from which NS-ICs are isolated can be a neural tissue, a population of cells dissociated from neural tissue, or a population of cells in cell culture, *e.g.*, cells in a neurosphere culture or an adherent neural stem cell culture.

The invention provides for the isolation and identification of NS-ICs. Identification of a neurosphere initiating stem cell or progenitor (NS-IC) involves contacting a population of neural cells (or a population which contains neural or neural derived cells) with a reagent that binds to the CD49f antigen and/or a reagent that binds to the CD133 antigen and/or a reagent that binds to the CD15 antigen, and detecting the contact between the reagent that binds to the CD49f and/or CD133 and/or CD15 antigens and the CD49f and/or CD133 and/or CD15 antigens on the surface

of cells. Those cells to which the CD49f and/or CD133 and/or CD15 reagents (*e.g.* $CD15^{hi}$ or $CD15^{-/lo}$ cells) bind are identified as NS-ICs. The identity of these cells can be confirmed by assays that demonstrate that the cells are in fact NS-ICs, capable of neurosphere initiation, self-renewal and multipotency.

The methods of this invention can also be used to isolate $CD49f^+$ cells from $CD49f^-$ cells using an anti-CD49f antibody (or $CD15^{hi}$ cells or $CD133^+CD15^{-/lo}CD24^{-/lo}$ cells using an anti-CD15 antibody), by combining a population of neural cells which contains a fraction of NS-ICs with a reagent that specifically binds to the CD49f antigen (or the CD15 antigen), and then selecting for $CD49f^+$ cells (or for $CD15^{hi}$ or $CD15^{-/lo}$ cells), to produce a selected population enriched in $CD49f^+$ NS-ICs (or to the $CD15^{hi}$ or $CD133^+CD15^{-/lo}CD24^{-/lo}$ NS-ICs) as compared with the population of neural cells prior to the selection. Accordingly, the invention further provides for the enrichment of NS-ICs from neural tissue or neural stem cell cultures (*e.g.*, neurosphere suspension cultures or neural stem cell adherent cultures). The invention is thus useful for the enrichment of NS-IC from neural tissue in which stem cells and progenitor cells occur at low frequency, or may have been depleted, such as late embryo, juvenile, and/or adult tissue. One of ordinary skill in the art can combine a population of neural cells containing a fraction of NS-ICs with a reagent that specifically binds to the CD49f antigen or the to CD15 antigen, and select for the $CD49f^+$, $CD15^+$, or $CD133^+CD15^{-/lo}CD24^{-/lo}$ cells. In this way, the selected $CD49f^+$, $CD15^+$, or $CD133^+CD15^{-/lo}CD24^{-/lo}$ cells are enriched in the fraction of NS-IC as compared with the population of neural cells.

The invention also provides antibodies that specifically binds to the CD49f antigen, wherein the CD49f antigen specifically binds to the GoH3 and/or 4F10 antibodies. This antibody may be produced by a hybridoma cell line. This monoclonal antibody may block simultaneous binding to the CD49f antigen by the antibody GoH3 and/or the antibody 4F10. Of particular interest are antibodies that bind to the CD49f antigen, cross-reactive antibodies (*i.e.*, those which bind to the same epitope as the GoH3 and/or 4F10 antibodies and substantially inhibit simultaneous binding), species analogs thereof, binding fragments thereof, and/or conjugates thereof.

Likewise, the invention also provides antibodies that specifically binds to the CD15 antigen, wherein the CD15 antigen specifically binds to the MMA antibody. This antibody may be produced by a hybridoma cell line. This monoclonal antibody may block simultaneous binding to the CD15 antigen by the antibody MMA. Of particular interest are antibodies that

bind to the CD15 antigen, cross-reactive antibodies (*i.e.*, those which bind to the same epitope as the MMA antibody and substantially inhibit simultaneous binding), species analogs thereof, binding fragments thereof, and/or conjugates thereof.

Also provided is a method for the further enrichment of human CNS-SC and progenitors which can initiate neurospheres (NS-IC) by combining a population of $CD49f^+$ or a population of $CD15^+$ or $CD15^{-/lo}$ neural or neural-derived cells with a reagent that specifically binds to the CD24 antigen and removing those cells that are $CD24^+$, wherein the remaining cells are enriched for NS-IC. For example, this reagent can be an antibody.

In any of the methods of this invention, the population of neural or neural-derived cells can be further enriched by contacting the cells with a reagent that specifically binds to the CD133 antigen (*i.e.*, an anti-CD133 antibody such as the AC133 monoclonal antibody) before, during, and/or after contacting the cells with a reagent that binds to the CD49f antigen. Likewise, in any of the methods of this invention, the population of neural or neural-derived cells can be further enriched by contacting the cells with a reagent that specifically binds to the CD15 antigen (*i.e.*, an anti-CD15 antibody) before, during, and/or after contacting the cells with a reagent that binds to the CD49f antigen.

Cell selection according to the invention can be accomplished by any suitable means known in the art, including flow cytometry, such as by fluorescence activated cell sorting using fluorochrome conjugated antibodies. The selection can also be by high gradient magnetic selection using antibodies conjugated to magnetic particles. Likewise, any other suitable method including attachment to and disattachment from solid phase, is also contemplated as being within the scope of the invention.

A population of cells can be derived by immunoselection using an anti-CD49f antibody. The population of cells should contain at least 30% $CD49f^+$ NS-ICs, preferably at least 50-70% $CD49f^+$ NS-ICs, and more preferably greater than 90% $CD49f^+$ NS-ICs. Most preferable would be a substantially pure population of $CD49f^+$ NS-ICs, containing at least 95% $CD49f^+$ NS-ICs. The degree of enrichment obtained, and actually used, depends on a number of factors, including the method of selection, the method of growth, and the cell dose of the cells that are placed in culture for the initiation of neurospheres.

The population of cells can be derived from late embryo, juvenile, or adult mammalian CNS tissue, or it may be derived from existing cultures of neural stem cells, as described in Weiss, United States patent 5,750,376, or Johe, United States patent 5,753,506. In the most

preferred embodiment, the NS-IC are human. In some embodiments, the CD49f$^+$ cells in the population can be complexed to endothelial cells.

The *in vitro* cell cultures described herein containing an enriched population of CD49f$^+$ NS-ICs are generally characterized as staining positive for nestin and, in the presence of differentiation-inducing conditions, produce progeny cells that differentiate into neurons, astrocytes, and oligodendrocytes.

One skilled in the art can introduce an isolated CD49f$^+$ cell to a culture medium, proliferate the isolated CD49f$^+$ cell in culture, particularly as a neurosphere; culture the progeny of the isolated CD49f$^+$ cell under conditions in which the isolated CD49f$^+$ cell differentiates to neurons, astrocytes, and oligodendrocytes; then detect the presence of neurons, astrocytes, and oligodendrocytes. The presence of neurons, astrocytes, and oligodendrocytes characterizes the isolated CD49f$^+$ cell as an NS-IC.

Typically, CD49f$^+$ NS-ICs are cultured in a medium that permits the growth and proliferation of neurospheres. The culture in which the isolated CD49f$^+$ cell proliferates can be a serum-free medium containing one or more predetermined growth factors effective for inducing multipotent neural stem cell proliferation. The culture medium can be supplemented with a growth factor selected from leukemia inhibitory factor (LIF), epidermal growth factor (EGF), basic fibroblast growth factor (FGF-2; bFGF) or combinations thereof. The culture medium can be further supplemented with neural survival factor (NSF) (Clonetics, CA). The conditions in which the CD49f$^+$ cell differentiates to neurons, astrocytes, and oligodendrocytes can include culturing the CD49f$^+$ cell progeny on a laminin-coated surface in culture medium containing fetal bovine serum (FBS) without EGF, FGF-2 or LIF.

The invention also provides a method for identifying the presence of a growth factor that affects the growth of NS-IC. One skilled in the art can combine a composition suspected of containing at least one growth factor that affects the growth of NS-IC with a composition containing NS-IC, then determine the growth of the NS-IC as a function of the presence of the composition. Altered (increased, decreased, *etc.*) NS-IC growth indicates the presence in the composition of a growth factor that affects the growth of NS-IC. The identity of the growth factor can be determined using techniques known in the art.

Antibodies to CD133. Antibodies to CD133 may be obtained or prepared as discussed in United States patent 5,843,633, incorporated herein by reference. The CD133 antigen can be contacted with an antibody, such as various anti-CD133 monoclonal antibodies (*e.g.*, AC133),

which have specificity for the CD133 antigen. "Anti-CD133 antibodies" are characterized by binding to the CD133 protein in native, in FACS, and immunoprecipitation experiments, as well as denatured, in Western blot experiments, conformations. The CD133 antigen has been reported to have molecular weights in the range of 125 kDa to 127 kDa according to United States patent 5,843,633 and 115 kDa to 127 kDa according to United States Published Patent Application No. 20010051372. The CD133 antigen is expressed on a subset of progenitor cells derived from human bone marrow, fetal bone marrow and liver, cord blood, and adult peripheral blood.

Antibodies to CD49f. Antibodies to CD49f may be obtained commercially or prepared according to methods known to those of ordinary skill in the art. The CD49f antigen can be contacted with an antibody, such as various anti-CD49f monoclonal antibodies, which have specificity for the CD49f antigen. Anti-CD49f antibodies are characterized by binding to the CD49f antigen under Western blot conditions from reducing SDS-PAGE gels. As used herein, the term "anti-CD49f antibody" refers to a monoclonal or polyclonal antibody that specifically binds to the CD49f antigen. Examples of anti-CD49f antibodies include, but are not limited to, GoH3 and 4F10. The CD49f antigen has a molecular weight, based on commercially available standards, in the range of about 140 kDa. The CD49f antigen is expressed on thymocytes, T lymphocytes, and monocytes. Increased expression is found on activated and memory T cells. The A splice variant alone is expressed in the lung, liver, spleen and cervix. The B splice variant alone is expressed in the brain, ovary, and kidney. Both forms are also detected in other tissues.

Antibodies to CD15. Antibodies to human CD15 may be obtained commercially or prepared according to methods known to those of ordinary skill in the art. The CD15 antigen can be contacted with an antibody, such as various anti-CD15 monoclonal antibodies, which have specificity for the CD15 antigen. Anti-CD15 antibodies are characterized by binding to the CD15 antigen under Western blot conditions from reducing SDS-PAGE gels. As used herein, the term "anti-CD15 antibody" refers to a monoclonal or polyclonal antibody that specifically binds to the CD15 antigen. Examples of anti-CD15 antibodies include, but are not limited to, MMA. The CD15 antigen has a molecular weight, based on commercially available standards, in the range of about 220 kDa. The CD15 antigen is expressed in mature human neutrophils, monocytes, and eosinophils. It can also be found present on embryonic tissues and adenocarcinomas, myeloid leukemias and Reed-Sternberg cells.

Preparation of antibodies. Antibodies to the CD133, CD49f and/or CD15 antigens can be obtained by immunizing a xenogeneic immunocompetent mammalian host (including murine,

rodentia, lagomorpha, ovine, porcine, bovine, *etc.*) with human progenitor cells. The choice of a particular host is primarily one of convenience. A suitable progenitor cell population for immunization can be obtained by isolating $CD34^+$ cells from cytokine mobilized peripheral blood, bone marrow, fetal liver, *etc.* In addition, a suitable progenitor cell population for immunization can be obtained from CNS neural stem cells or other NS-IC. Immunizations are performed in accordance with conventional techniques, where the cells may be injected subcutaneously, intramuscularly, intraperitoneally, intravascularly, *etc.* Normally, from about 10^6 to 10^8 cells are used, which may be divided into one or more injections, usually not more than about 8 injections, over a period of from about one to about three weeks. The injections may be with or without adjuvant, *e.g.* complete or incomplete Freund's adjuvant, specol, alum, *etc.*

After completion of the immunization schedule, the antiserum may be harvested in accordance with conventional methods to provide polygonal antisera specific for the surface membrane proteins of progenitor cells, including the CD133, CD49f and/or CD15 antigens. Lymphocytes are harvested from the appropriate lymphoid tissue, *e.g.* spleen, draining lymph node, *etc.*, and fused with an appropriate fusion partner, usually a myeloma line, producing a hybridoma secreting a specific monoclonal antibody. Screening clones of hybridomas for the antigenic specificity of interest is performed in accordance with conventional methods.

The anti-CD133, anti-CD49f and/or anti-CD15 antibodies can be produced as a single chain, instead of the normal multimeric structure. Single chain antibodies are described in *e.g.*, Jost *et al.*, 269 J. BIOL. CHEM. 26267-73 (1994), incorporated herein by reference. DNA sequences encoding the variable region of the heavy chain and the variable region of the light chain are ligated to a spacer encoding at least about 4 amino acids of small neutral amino acids, including glycine or serine. The protein encoded by this fusion allows assembly of a functional variable region that retains the specificity and affinity of the original antibody. Anti-CD133, anti-C49f and/or anti-CD15 antibodies can also be produced by use of Ig cDNA for construction of chimeric immunoglobulin genes (Liu *et al.*, 84 PROC. NATL. ACAD. SCI. 3439 (1987) and 139 J. IMMUNOL. 3521 (1987), incorporated herein by reference. mRNA is isolated from a hybridoma or other cell producing the antibody and used to produce cDNA. The cDNA of interest may be amplified by the polymerase chain reaction using specific primers (U.S. Patent 4,683,195 and U.S. Patent 4,683,202).

Alternatively, a library is made and screened to isolate the sequence of interest. The DNA sequence encoding the variable region of the antibody is then fused to human constant region

sequences. The sequences of human constant regions genes may be found in Kabat *et al.*, "Sequences of Proteins of Immunological Interest" N.I.H. PUBLICATION NO. 91-3242 (1991). Human C region genes are readily available from known clones. The chimeric, humanized antibody is then expressed by conventional methods.

Anti-CD133, anti-CD49f and/or anti-CD15 antibodies can also be produced as antibody fragments, such as Fv, $F(ab')_2$ and Fab. Antibody fragments may be prepared by cleavage of the intact protein, *e.g.* by protease or chemical cleavage. Alternatively, a truncated gene can be designed. For example, a chimeric gene encoding a portion of the $F(ab')_2$ fragment would include DNA sequences encoding the CH1 domain and hinge region of the H chain, followed by a translational stop codon to yield the truncated molecule.

Immunostaining. Biological samples are assayed for the presence of $CD133^+$, $CD49f^+$ and/or $CD15^+$ or $CD15^{-/lo}$ cells by any convenient immunoassay method for the presence of cells expressing the surface molecule bound by the subject antibodies. Assays may be performed on cell lysates, intact cells, frozen sections, *etc.* Any commercially available antibodies are suitable for the direct immunofluorescent staining of cells.

Cell sorting. The use of cell surface antigens found on NS-IC cells provides a means for the positive immunoselection of progenitor cell populations, as well as for the phenotypic analysis of progenitor cell populations using flow cytometry. Cells selected for expression of CD49f and/or CD15 antigen may be further purified by selection for other stem cell and progenitor cell markers, including CD133.

For the preparation of substantially pure progenitors and stem cells, a subset of progenitor cells is separated from other cells on the basis of CD49f and/or CD15 binding. Progenitors and stem cells may be further separated by binding to other surface markers known in the art, including CD133. Selection of $CD133^+$ cells may be accomplished before, during or after selection of $CD49f^+$ and/or $CD15^+$ or $CD15^{-/lo}$ cells. Likewise, selection of $CD15^+$ or $CD15^{-/lo}$ cells may be accomplished before, during or after selection of $CD49f^+$ and/or $CD133^+$ cells. Procedures for separation may include magnetic separation, using antibody-coated magnetic beads, affinity chromatography and "panning" with antibody attached to a solid matrix, *e.g.* plate, or other convenient technique. Techniques providing accurate separation include fluorescence activated cell sorters, which can have varying degrees of sophistication, such as multiple color channels, low angle and obtuse light scattering detecting channels, impedance channels, *etc.* Dead cells may be eliminated by selection with dyes associated with dead cells (propidium iodide

[PI], LDS). Any technique, which is not unduly detrimental to the viability of the selected cells, known to those in the art may be employed.

Conveniently, the antibodies are conjugated with labels to allow for ease of separation of the particular cell type, *e.g.* magnetic beads; biotin, which binds with high affinity to avidin or streptavidin; fluorochromes, which can be used with a fluorescence activated cell sorter; haptens; and the like. Multi-color analyses may be employed with the FACS or in a combination of immunomagnetic separation and flow cytometry. Multi-color analysis is of interest for the separation of cells based on multiple surface antigens, *e.g.* $CD49f^{+}CD24^{-/lo}$, $CD49f^{-}CD24^{+}$, $CD15^{+}CD24^{-/lo}$, $CD15^{-/lo}CD24^{+}$, $CD15^{-/lo}CD24^{-/lo}$, $CD133^{+}CD49f^{+}CD24^{-/lo}$, $CD133^{-}CD49f^{-}CD24^{+}$, $CD133^{+}CD15^{+}CD24^{-/lo}$, $CD133^{-}CD15^{-/lo}CD24^{+}$, $CD133^{+}CD15^{-/lo}CD24^{-/lo}$, $CD133^{+}CD49f^{+}CD15^{+}CD24^{-/lo}$, $CD133^{-}CD49f^{-}CD15^{-/lo}CD24^{+}$, $CD133^{+}CD49f^{+}CD15^{-/lo}CD24^{-/lo}$, *etc.*

Fluorochromes, which find use in a multi-color analysis include phycobiliproteins, *e.g.* phycoerythrin and allophycocyanins; fluorescein and Texas red. A negative designation indicates that the level of staining is at or below the brightness of an isotype matched negative control. A dim or low designation indicates that the level of staining may be near the level of a negative stain, but may also be brighter than an isotype matched control.

In one embodiment, the anti-CD133, anti-CD49f and/or anti-CD15 antibodies are directly or indirectly conjugated to a magnetic reagent, such as a superparamagnetic microparticle (microparticle). Direct conjugation to a microparticle can be achieved by use of various chemical linking groups, as known in the art. The antibody can be coupled to the microparticles through side chain amino or sulfhydryl groups and heterofunctional cross-linking reagents. A large number of heterofunctional compounds are available for linking to entities. A preferred linking group is 3-(2-pyridyidithio)propionic acid N-hydroxysuccinimide ester (SPDP) or 4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester (SMCC) with a reactive sulfhydryl group on the antibody and a reactive amino group on the magnetic particle.

Alternatively, the anti-CD133, anti-CD49f and/or anti-CD15 antibodies can be indirectly coupled to the magnetic particles. The antibody is directly conjugated to a hapten, and hapten-specific, second stage antibodies are conjugated to the particles. Suitable haptens include digoxin, digoxigenin, FITC, dinitrophenyl, nitrophenyl, avidin, biotin, *etc.* Methods for conjugation of the hapten to a protein are known in the art, and kits for such conjugations are commercially available.

To practice the methods of the invention, the anti-CD49f and/or anti-CD15 antibodies are added to a cell sample. The amount of anti-CD49f and/or anti-CD15 antibody necessary to bind a particular cell subset is empirically determined by performing a test separation and analysis. The cells and anti-CD49f and/or anti-CD15 antibodies are incubated for a period of time sufficient for complexes to form, usually at least about 5 minutes, more usually at least about 10 minutes, and usually not more than one hour, more usually not more than about 30 minutes.

The cells may additionally be incubated with antibodies or binding molecules specific for cell surface markers known to be present or absent on progenitor or stem cells. For example, the cells can be incubated with an anti-CD133 antibody either prior to, during, or after incubation with an anti-CD49f and/or anti-CD15 antibody to produce a further enriched population of NS-IC. The cells can be incubated with an anti-CD15 antibody either prior to, during, or after incubation with an anti-CD49f and/or anti-CD133 antibody to produce a further enriched population of NS-IC. The labeled cells are separated in accordance with the specific antibody preparation. Fluorochrome labeled antibodies are useful for FACS separation, magnetic particles for immunomagnetic selection, particularly high gradient magnetic selection (HGMS), *etc.* Exemplary magnetic separation devices are described in WO 90/07380, PCT/US96/00953, and EP 438,520, each of which is incorporated herein by reference. The AC133 Cell Isolation Kit (Miltenyi Biotec Inc., Auburn CA) can be used for the positive selection of $AC133^+$ cells. The kit provides a tool for single step isolation of $AC133^+$ cells (*i.e.*, cells that have the CD133 antigen. The AC133 Cell Isolation Kit contains FcR Blocking Reagent and MACS colloidal MicroBeads conjugated to the monoclonal mouse anti-human AC133 antibody.

The purified cell population may be collected in any appropriate medium. Various commercially available media may be used, including Dulbecco's Modified Eagle Medium (DMEM), Hank's Basic Salt Solution (HBSS), Dulbecco's phosphate buffered saline (DPBS), RPMI, Iscove's modified Dulbecco's medium (IMDM), phosphate buffered saline (PBS) with 5 mM EDTA, *etc.*, frequently supplemented with fetal calf serum (FCS), bovine serum albumin (BSA), human serum albumin (HSA), *etc.*

Populations highly enriched for human progenitor or stem cells are achieved in this manner. The desired cells will be 30% or more of the cell composition, preferably 50% or more of the cell population, more preferably 90% or more of the cell population, and most preferably 95% or more (*e.g.* substantially pure) of the cell population.

Use of purified stem cell/progenitor cells. $CD133^+$ $CD49f^+$, $CD133^+CD15^+$,

$CD133^+CD15^{-/lo}$, $CD133^+CD49f^+CD15^+$, $CD133^+CD49f^+CD15^{-/lo}$, $CD15^{-/lo}CD49f^+$, and/or $CD15^+CD49f^+$ stem cells/progenitor cells are useful in a variety of ways. The $CD133^+CD49f^+$, $CD133^+CD15^+$, $CD133^+CD15^{-/lo}$, $CD133^+CD49f^+CD15^+$, $CD133^+CD49f^+CD15^{-/lo}$, $CD15^{-/lo}CD49f^+$, and/or $CD15^+CD49f^+$ cells can be used to reconstitute a host whose cells have been lost through disease or injury. Genetic diseases associated with cells may be treated by genetic modification of autologous or allogeneic stem cells to correct a genetic defect or treat to protect against disease. Alternatively, normal allogeneic progenitor cells may be transplanted. Diseases other than those associated with cells may also be treated, where the disease is related to the lack of a particular secreted product such as hormone, enzyme, growth factor, or the like. CNS disorders encompass numerous afflictions such as neurodegenerative diseases (*e.g.* Alzheimer's and Parkinson's), acute brain injury (*e.g.* stroke, head injury, cerebral palsy) and a large number of CNS dysfunctions (*e.g.* depression, epilepsy, and schizophrenia). In recent years neurodegenerative disease has become an important concern due to the expanding elderly population, which is at greatest risk for these disorders. These diseases, which include Alzheimer's Disease, Multiple Sclerosis (MS), Huntington's Disease, Amyotrophic Lateral Sclerosis, and Parkinson's Disease, have been linked to the degeneration of neural cells in particular locations of the CNS, leading to the inability of these cells or the brain region to carry out their intended function. By providing for maturation, proliferation and differentiation into one or more selected lineages through specific different growth factors the progenitor cells may be used as a source of committed cells. Neurospheres can also be used to produce a variety of blood cell types, including myeloid and lymphoid cells, as well as early hematopoietic cells (*see,* Bjornson *et al.,* 283 SCIENCE 534 (1999), incorporated herein by reference).

The $CD133^+$ $CD49f^+$, $CD133^+CD15^+$, $CD133^+CD15^{-/lo}$, $CD133^+CD49f^+CD15^+$, $CD133^+CD49f^+CD15^{-/lo}$, $CD15^{-/lo}CD49f^+$, and/or $CD15^+CD49f^+$ cells may also be used in the isolation and evaluation of factors associated with the differentiation and maturation of cells. Thus, the cells may be used in assays to determine the activity of media, such as conditioned media; to evaluate fluids for growth factor activity, involvement with dedication of lineages, or the like.

The $CD133^+$ $CD49f^+$, $CD133^+CD15^+$, $CD133^+CD15^{-/lo}$, $CD133^+CD49f^+CD15^+$, $CD133^+CD49f^+CD15^{-/lo}$, $CD15^{-/lo}CD49f^+$, and/or $CD15^+CD49f^+$ cells may be frozen at liquid nitrogen temperatures and stored for long periods of time, being thawed and capable of being reused. The cells will usually be stored in 5% DMSO and 95% fetal calf serum. Once thawed, the

cells may be expanded by use of growth factors or stromal cells associated with stem cell proliferation and differentiation.

The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. These examples should in no way be construed as limiting the scope of the invention, as defined by the appended claims.

EXAMPLE 1
ISOLATION OF NS-IC BY DIFFERENT MARKERS

The CD24 antigen, recognized by for example, the SC20 (8G1) monoclonal antibody, can also be evaluated as a subselector for neural stem cells. Cells that are $CD24^{-/lo}$ ($8G1^{-/lo}$) display more stem cell-like properties, while cells that are $CD24^{med/hi}$ ($8G1^{med/hi}$) display more progenitor cell-like properties (Figure 3), in isolates of fresh fetal brain.

Following culture, CD24 expression can be upregulated depending on cell cycle status, days post last passage, and culture conditions. Thus, long-term neurosphere cells derived from $CD24^{-/lo}$ fetal brain cells become heterogeneous for CD24 expression.

EXAMPLE 2
NEUROSPHERE INITIATING CELLS CAN BE SEPARATED BASED ON CD49f EXPRESSION: FLOW CYTOMETRY CELL SORTING (FACS) APPROACH

The purpose of this EXAMPLE is to test whether $CD49f^{+}$ cells are the only cells in the brain that have pluripotent NSC activity. To measure neural stem cells and primitive progenitor activities, a NS-IC assay will be established to determine frequency of NS-IC in a given population. When NS-IC are rare and express CD49f antigen, NS-IC can be enriched by $CD49f^{+}$ selection, and correspondingly depleted in other fractions.

Source of monoclonal antibodies: CD49f antigen is recognized by at least the following monoclonal antibodies: GoH3 (Research Diagnostics, Inc. (Flanders, NJ); BD Biosciences (www.bdbiosciences.com); and ICN Biomed (www.incbiomed.com)) and 4F10 (Research Diagnostics, Inc. (Flanders, NJ)).

Human fetal brain (FBR 10-20 gestational week ["g.w"]) are obtained after obtaining informed consent. Human fetal brain tissues are cut into 1-3 mm cubed pieces using scalpels,

transferred into 50mL centrifuge tube and washed once with 0.02% EDTA/PBS solution. Tissue pieces are dissociated enzymatically in the presence of collagenase and hyaluronidase at 37 degrees or 1 hour.

During the next days, the dissociated tissue pieces are washed once with 0.02% EDTA/PBS solution and dissociated enzymatically in the presence of trypsin at 37 degrees for 15 minutes. Debris and aggregates are removed by filtering cell suspensions through 70 micron filter cup. Typically 1-10 x 10^8 cells were obtained from each FBr tissue, 16-20 gestational wk. Cells were resuspended in HBSS buffer containing 0.1 % human serum albumin and 10mM HEPES.

The staining and sorting of CNS-SC from FBr were performed as follows. Typically the dissociated FBr cells were incubated and stained with mAb against CD133, CD24-FITC, and CD49f-PE. Stained cells were washed and resuspended in HBSS containing 0.1 % human serum albumin, 10mM HEPES (Gibco) and 0.5μg/mL of propidium iodine (PI) and sorted with a dual-laser Vantage SE (BDIS).

$CD49f^+$ $CD24^{-/lo}$ FACS separated cells are cultured in typically, X vivo 15 or combination of X vivo 15, D-MEM,/F-12 media is used as a basal media. To maximize neurosphere development, the sorted cells are typically cultured in the presence of LIF, FGF-2, EGF, as described in Example 3, *infra*. Neurosphere cells established from $CD49F^+$ $CD24^{-/lo}$ sorted cells will express nestin, as can be tested after approximately 7 days in culture and can be detected mouse anti-human nestin antibody (Chemicon). For example, the neurosphere cells derived from $CD133^+$ $CD24^{-/lo}$ sorted CNS-SC available from StemCells Inc. (Palo Alto, CA) express nestin. After expansion, the expanded neurosphere cells were transplanted into neonatal NOD-SCID mice as described. They displayed robust engraftment as equivalent as we observed from neurosphere cells derived from CD133+ CD24-/lo CNS-SC. When induced to differentiate, the $CD49f^+$ $CD24^{-/lo}$ sorted/expanded neurosphere cells could differentiate into neurons in morphology, which express b-tubulin III and mature astrocytes morphology which express GFAP. In this particular differentiation assay, neurosphere cells will be cultured onto a poly-ornithine coated surface in the presence of 0-1% FBS, BDNF, GDNF or Epo without EGF, FGF-2 and LIF.

Other differentiation assays can be used to induce differentiation of NS-IC to neurons, astrocytes and oligodendrocytes.

The quantitative NS-IC assay can be performed on unpurified tissue cells, on $CD49f^+$ sorted cells, and on clonal neurosphere cell lines.

EXAMPLE 3
CELL CULTURE MEDIA FOR GROWTH AND PASSAGE OF NS-IC

Weiss *et al.*, U.S. Patent 5,750,376 and Weiss *et al.*, US Patent 5,851,832 disclose "culture medium containing one or more predetermined growth factors effective for inducing multipotent neural stem cell proliferation" and "differentiation-inducing conditions". However, different basal media can be used, including, but not limited to:

D-MEM/F12 (Gibco BRL, Gaithersburg, MD);

X vivo 15 (Bio Whittaker, Walkersville, MD);

Neural progenitor basal media, (Clonetics. San Diego, CA); or

combinations of the basal media listed above.

A typical media formulation to culture human neurosphere cells is provided in TABLE 1.

TABLE 1
Serum-Free N2/EGF Supplemented Culture Medium For Neurospheres

Quantity	Reagents
87 ml	DMEM/F12 (Gibco lot. 1012915; Cat. No. 11330-032)
1 ml	N-2 Supplement (Gibco lot 1017018; Cat. No. 17502-014)
1 ml	0.2 mg/ml heparin (Sigma lot 28H0320; Cat. No. H-3149)
1 ml	0.2 M Glutamine (JCR lot 7N2320; Cat. No. 59202-77p)
10 ml	3 % Glucose (Sigma, lot 37H0841; Cat. No. G-7021)
20 µl	100 µg/ml EGF (R&D lot CE107091; Cat. No. 236-EG)
100 µl	20 µg/ml FGF-2 (Gibco lot KCQ411; Cat. No. 13256-029)
100 µl	10µg/ml LIF (R&D lot OX038021; Cat. No. 250-L)

EGF is added to 100 ml base medium for human neurospheres after filtering the medium. EGF is relatively stable in the medium. FGF-2 and LIF are added when medium is ready to use. The final concentrations of the supplement reagents are:

5 μg/ml	Insulin
100 μg/ml	Human transferrin
6.3 ng/ml	Progesterone
16.1 μg/ml	Putrascine
5.2 ng/ml	Selenite
20 ng/ml	EGF
20 ng/ml	FGF-2
10 ng/ml	LIF
2 μg/ml	Heparin
2 mM	L-glutamine
6 mg/ml	Glucose

The optimization of media formulation permits a higher percentage of neurospheres initiated from primary brain tissue to be established. X vivo 15 media is preferred. The optimization of media formulation also permits a more consistent growth of neurospheres.

EXAMPLE 4
CD49f IS A CRITICAL CELL SURFACE MARKER EXPRESSED ON CELLS FROM LONG-TERM NEUROSPHERE CULTURE

A long-term neurosphere cells culture can be obtained from StemCells, Inc (Palo Alto, CA). The majority of cells express CD133 (>90%) and CD49f (>80%). When X Vivo 15 is used as basal media, higher percentage of neurosphere cultures initiated from 18 g.w. It is therefore possible to evaluate CD49f cells increases as neurosphere develops. Once neurosphere cells are well established, virtually all cells forming neurospheres express CD133 and CD49f (Figure 5).

EXAMPLE 5
NEUROSPHERE-INITIATING CELLS (NS-IC) CAN BE SEPARATED BASED ON CD15 EXPRESSION: FLOW CYTOMETRY CELL SORTING (FACS) APPROACH TO ISOLATE $CD15^{+}CD24^{-/lo}$ and $CD133^{+}CD15^{-/lo}CD24^{-/lo}$ FETAL BRAIN CELLS

The purpose of this EXAMPLE is to test whether $CD15^{hi}$ cells are the only cells in the brain that have pluripotent NSC activity. To measure neural stem cells and primitive progenitor activities, a NS-IC assay is established to determine frequency of NS-IC in a given population. When NS-IC are rare and express CD15 antigen, NS-IC can be enriched by $CD15^{+}$ selection, and correspondingly depleted in other fractions.

Source of monoclonal antibodies: CD15 antigen is recognized by at least the following monoclonal antibody: MMA (BD Biosciences (www.bdbiosciences.com) (catalog numbers 340703, 340850, 347420, 347423, 559045)).

Human fetal brain (FBR 10-20 gestational week ["g.w"]) were obtained after obtaining informed consent. Human fetal brain tissues are cut into 1-3 mm cubed pieces using scalpels, transferred into 50mL centrifuge tube and washed once with 0.02% EDTA/PBS solution. Tissue pieces were dissociated enzymatically in the presence of collagenase and hyaluronidase at 37 degrees or 1 hour and stored over night at 4 degrees.

During the next days, the dissociated tissue pieces were washed once with 0.02% EDTA/PBS solution and dissociated enzymatically in the presence of trypsin at 37 degrees for 15 minutes. Debris and aggregates are removed by filtering cell suspensions through 70 micron filter cup. Typically 1-10 x 10^8 cells were obtained from each FBr tissue, 16-20 gestational wk. Cells were resuspended in HBSS buffer containing 0.1 % human serum albumin and 10mM HEPES.

The staining and sorting of CNS-SC from FBr are performed as follows. Typically the dissociated FBr cells are incubated and stained with mAb against CD133, CD15, and CD24. Stained cells are washed and resuspended in HBSS containing 0.1 % human serum albumin, 10mM HEPES (Gibco) and 0.5μg/mL of propidium iodine (PI) and sorted with a dual-laser Vantage SE (BDIS).

NS-IC activity is highly enriched in the both CD133+ $CD15^{hi}$ CD24-/lo and CD133+ CD15-/lo CD24-/lo cell population (Figure 7). Virtually no NI-IC cells were detected from CD15-/lo CD24 hi cell population

$CD15^{hi}CD24^{-/lo}$ FACS separated cells are cultured in X vivo 15 or combination of X vivo 15, D-MEM,/F-12 media is used as a basal media. To maximize neurosphere development, the sorted cells are typically cultured in the presence of LIF, FGF-2, EGF, as described in Example 3. Neurosphere cells established from $CD15^{hi}CD24^{-/lo}$ sorted cells will express nestin, as can be tested after approximately 7 days in culture and can be detected with mouse anti-human nestin antibody (Chemicon). For example, the neurosphere cells available from StemCells Inc. (Palo Alto, CA) express nestin. When induced to differentiate, the $CD15^{hi}CD24^{-/lo}$ sorted/expanded neurosphere cells can be differentiated into neurons in morphology which express b-tubulin III and mature astrocytes morphology which express GFAP. In this particular differentiation assay, neurosphere cells will be cultured onto a poly-

ornithine coated surface in the presence of 0-1% FBS, BDNF, GDNF or Epo without EGF, FGF-2 and LIF.

Other differentiation assays can be used to induce differentiation of NS-IC into neurons, astrocytes and oligodendrocytes. Upon differentiation, $CD133^+$ $CD15^{-/lo}CD24^{-/lo}$ cells are multipotential (Figure 6).

EXAMPLE 6
TRANSPLANTATION OF $CD49f^+CD24^{-/lo}$ and $CD133^+CD15^{-/lo}$ $CD24^{-/lo}$ SORTED/EXPANDED NEUROSPHERE CELLS INTO NEONATAL NOD-SCID MICE

NOD SCID mice have provided an excellent model system for the engraftment of a number of different human cell types including the hematopoietic stem cell. Expanded $CD49f^+CD24^{-/lo}$ or $CD133^+CD15^{-/lo}CD24^{-/lo}$ neurosphere cells at passages 6-10 are harvested and gently dissociated with collagenase. Neonatal mice (P0-P1) are anesthetized by placing them in ice for 5-10 minutes. Once cryo-anesthetized, the pups are placed on a stereotaxic device and injected with 1-2 ul of cells ranging from 10^5-10^6 cells/injection into the lateral ventricle. The injected mice are kept 18-27 weeks prior to testing the engraftment of human cells.

Generation of human specific monoclonal antibodies for tracking human cells in vivo

Human CNS-SC neurospheres were transplanted into the lateral ventricle of NOD-Scid neonatal mice. Newborn (P0-P1) mice were injected with 10^5 cells/site into each lateral ventricle. Human cell engraftment was assessed 1-10 months after transplantation by immunohistochemistry.

Due to inter-species conservation in the sequence of proteins used to characterize neural cells (>90% homology in many cases), most commercially available monoclonal antibodies (mAbs) against neural cells (e.g. β-tubulin III, GFAP, MBP) recognize their antigens in the mouse, rat, primate and human. Extensive testing of commercially available mAbs failed to identify any that would distinguish human cells in a xenogeneic recipient. Therefore, a panel of human specific mAbs was generated at SCI that has been invaluable for the assessment of engraftment and migration of human cells. Extensive testing on mouse and rat brains confirmed that they do not cross-react with their neural cells. Among the SC121 has been routinely and reproducibly used as a marker of human cell engraftment in NOD-Scid recipients. Western blot analysis indicates that SC121 recognizes a 25 kDA protein found in human cells but absent from mouse cells. Immunohistochemical staining with non-transplanted rat brains show there is no cross reactivity

in rat brains as well. StemCells Inc. has generated human specific mAbs that distinguish specific neural lineages such as neurons, astrocytes and oligodendrocytes (Table 3). These human specific reagents have been invaluable for the assessing engraftment, migration and differentiation of human cells. For quantitation of human engraftment, it is possible to perform in situ hybridization using human specific DNA probe for Alu-1 repeats.

Table 3. mAb and DNA probe for monitoring hCNS-SC

Name	Antigen	Source	Specificity
SC101	Human nuclei	StemCells Inc.	A subset of human nuclei
SC112	N-CAM	StemCells Inc.	Human neuronal lineage
SC121	Cytoplasmic protein	StemCells Inc.	Pan-human
SC123	Human GFAP	StemCells Inc.	Human GFAP+ cells
Alu-1	DNA probe in situ	StemCells Inc.	All human nuclei

Six to 36 weeks post-transplantation, the injected mice are perfused with 4% paraformaldehyde. The mouse brains are sectioned sagitally at 40 um thickness. To detect transplanted human cells, sections will be incubated with mAb SC121 (StemCells, Inc.), followed by incubation with a biotinylated goat anti-mouse IgG and the components of the VECTASTAIN ELITE ABC kit, using the methods employed in preliminary studies. The antibody-immunoperoxidase complex will be detected using the NovaRED substrate (Vector, Burlingame, CA).

To evaluate *in vivo* engraftment migration and the differentiation capacity of hCNS-SC, 10^5 cells from $CD49f^+CD24^{-/lo}$ or $CD133^+$ $CD15^+CD24^{-/lo}$ sorted/expanded neurosphere cultures at passage 7-10 can be injected into the lateral ventricles of neonatal NOD-SCID mice. Similar to CD133+ CD24-/lo sorted/expanded neurosphere cells, they engrafted robustly, migrate into olfactory bulb and hippocampus, and differentiate into neuron and glia morphologically (Figure 7).

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OTHER EMBODIMENTS

It is to be understood that, while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.